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Clinical significance of overexpression of NRG1 and its receptors, HER3 and HER4, in gastric cancer patients

위암에서 Neuregulin1과
수용체 HER3와 HER4의
발현양상 및 임상적 의의

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ABSTRACT

Clinical significance of overexpression of NRG1 and its receptors, HER3 and HER4, in gastric cancer patients

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Neuregulin 1 (NRG1), a ligand for human epidermal growth factor (HER) 3 and HER4, can activates cell signaling pathways to promote carcinogenesis and metastasis. To investigate the clinicopathologic significance of NRG1 and its receptors, immunohistochemistry was performed for NRG1, HER3, and HER4 in 502 consecutive gastric cancers (GCs). Furthermore, HER2, microsatellite instability (MSI), and Epstein-Barr virus (EBV) status were investigated. *NRG1* gene copy number (GCN) was determined by dual-color fluorescence *in situ* hybridization (FISH) in 388 available GCs. NRG1 overexpression was observed in 141 (28.1%) GCs and significantly associated with aggressive features, including infiltrative tumor growth, lymphovascular, and neural invasion, high pathologic stage, and poor prognosis (all $P < 0.05$),

but not associated with EBV, MSI, or HER2 status. HER3 cytoplasmic and membranous expression were observed in 157 (31.3%) and 13 (2.6%), respectively. HER4 cytoplasmic expression was observed in 277 (55.2%), including 115 (22.9%) cases with nuclear expression. In contrast to NRG1, cytoplasmic expression of HER3 and HER4 proteins were not associated with survival, but GC patients with HER3 membranous expression showed significantly worse survival. In addition, HER4 nuclear expression was inveresely correlated with patients outcome in GC. NRG1 overexpression was also closely correlated with HER3 (P = 0.034) and HER4 (P < 0.001) cytoplasmic expression. *NRG1* GCN gain (GCN ≥ 2.5) was detected in 14.7% patients, including two cases of amplification, and was moderately correlated with NRG1 overexpression (κ, 0.459; P < 0.001). Multivariate analysis identified NRG1 overexpression as an independent prognostic factor for survival (P = 0.040), unlike HER3 and HER4 expression. In 14 HER2 positive GC with trastzumab combined chemotherapy, coexpression of NRG1 and HER3 was detected in 2 (14.3%) cases, and these GC patients group with coexpression of NRG1 and HER3 also showed a shorter PFS (P = 0.005). Although our results indicate a lack of prognostic significance of HER3 and HER4 overexpression in GC, overexpression of their ligand, NRG1, was associated with aggressive clinical features and represented an independent unfavorable prognostic factor. Therefore, NRG1 is a potential prognostic and therapeutic biomarker in GC patients.

Keywords **:** Gastric cancer, Neuregulin1, Immunohistochemistry, Fluorescence *in situ* hybridization, Copy number gain

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INTRODUCTION

Despite recent diagnostic and therapeutic advances, gastric cancer (GC) remains a leading cause of cancer deaths, particularly in South Korea [1]. Deeper understanding of the molecular pathogenesis of GC has contributed to successful clinical application of targeted drugs, for example, drugs targeting to human epidermal growth factor receptor (HER) 2 mutations [2]. The HER family consists of four transmembrane proteins, HER1 (EGFR), HER2, HER3, and HER4. HER2 is well studied and can induce cell proliferation, differentiation, and apoptosis [2]. HER2 overexpression has been found in a subset (20–30%) of GC samples, primarily as a result of *HER2* gene amplification [2,3], and currently, drugs targeting HER2-positive GC are increasingly used as part of treatment for patients with advanced GC, as they can significantly improve outcomes [3, 4]. Unfortunately, a significant number of these patients eventually develop drug resistance and exhibit poor survival rates [4, 5]; hence, recent studies have focused on other members of the HER family, including HER3 and HER4 and their ligands.

Neuregulin (NRG) is a ligand of HER family protein, which has more than 32 isoforms. NRG1 is the predominant ligand of HER3 and HER4. Through binding to HER3, it functions in specific regulation of cell proliferation and organ development [6, 7]. Additionally, NRG1 can induce carcinoma development and promote metastasis [7]. Interestingly, recent studies have suggested that PI3K/Akt activation through the NRG1/HER3 signaling pathway leads to development of resistance to HER2-targeted treatment, and

it has been proposed that inhibition of this signaling pathway has potential as a therapeutic option to overcome resistance to anti-HER2 treatment [8–11]. However, few studies have assessed the association of NRG1 status and GC or the clinicopathologic significance of the NRG1/HER3/HER2 and NRG1/HER4/HER2 axis in GC.

Unlike other HER family proteins, HER3 lacks significant tyrosine kinase activity; it has a regulatory function through heterodimer formation with other members of the HER family [12]. Heterodimer containing HER3 can activate the following two key signaling pathways: mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt [12]. In various cancers, HER3/HER2/PI3K/Akt signaling promotes tumor cell proliferation and survival [6, 12, 13]. Several studies have demonstrated associations between HER3 protein expression and poor survival in various cancers including GC [14–17].

HER4 has markedly different functions in tumors, including functionally distinct splice isoforms and multiple proteolytically derived types. Alternative splicing of HER4 releases its intracellular domain and enables it to translocate to the nucleus [18–20]. Although the function of nuclear HER4 has not been fully elucidated, it has a role as a transcriptional cofactor [19]. Several previous studies have reported various prognostic associations with HER4 immunohistochemistry (IHC) results, particularly in breast cancer, including a correlation between cytoplasmic HER4 and improved prognosis [18].

However, the prognostic role of cytoplasmic and nuclear expression of HER4 in GC remains unclear. Moreover, detailed information regarding the mechanism of action of HER4 and its relationship with its ligand in GC is lacking [17].

In this study, we aimed to determine the prevalence and clinicopathologic implications of NRG1 expression in a large cohort of GC samples and to assess the relationship between NRG1 expression and that of HER3 and HER4. In addition, NRG1 expression status in GC was compared with HER2 positivity, Epstein-Barr virus (EBV) *in situ* hybridization (ISH), and microsatellite instability (MSI) status. We evaluated the *NRG1* gene copy number (GCN) status using dual-color fluorescence *in situ* hybridization (FISH) analysis and compared the concordance rate between protein expression and genetic alteration for NRG1.

MATERIALS AND METHODS

Patients and clinicopathological characteristics

A total of 502 consecutive GC patients who had curative surgery at Seoul National University Bundang Hospital from May 2003 to December 2005 were analyzed in this study. Clinical information including age, sex, size, location, and pathologic stage were collected from medical records retrospectively. Patients who had received preoperative chemotherapy or radiotherapy were excluded from this study. The American Joint Committee on Cancer seventh staging system was used to determine pTNM stage [21]. Disease-specific survival (DSS) data were collected, including patient outcome, the interval between the date of surgery and the date of death due to GC, and the period of disease-free survival (DFS) from surgery until the date of disease progression, death, or last disease assessment. In addition, to evaluate the prognostic impact of NRG1/HER3 signaling for HER2 targeting treatment, we collected 14 HER2 positive GC patients who had surgical operation and received chemotherapy combined with trastzumab at Seoul National University Bundang Hospital from 2009 to 2012.

Tissue microarray (TMA) construction

TMA blocks were constructed for 502 cases of GC, using previously described methods [22]. Briefly, we selected a representative tumor area for TMA construction in each case, and tissue cores of 2 mm diameter were transferred to the TMA block. Samples were considered valid when the tumor

occupied more than 15% of the core area. Serial sections were cut and used for IHC and FISH analyses.

Immunohistochemistry

We performed IHC using anti-NRG1 (1:2000, Abcam, Cambridge, MA, USA), anti-HER3 (1:3000, Thermo Scientific, Fremont, CA, USA), anti-HER4 (1:8000, Thermo scientific), and anti-HER2 (4B5; pre-dilution; Ventana, Medical Systems, Tucson, AZ, USA) antibodies with a Ventana Benchmark automatic immunostaining system (BenchMark XT, Ventana Medical system), according to the manufacturer's instructions. Antigen retrieval for immunohistochemistry consisted of Cell Conditioning 1 (CC1) (pH 8.4) for 24 min at 100 ℃. Sections on microslides were incubated with these antibodies and immunoreactivity detected using diaminobenzidine (DAB) substrate. Immunostaining was interpreted without prior knowledge of clinicopathologic data. NRG1, HER3, and HER4 were faintly expressed in the foveolar glands of non-neoplastic gastric mucosa; however, weak to moderate expression was observed in the cytoplasm of deep gastric glands (Fig. 1). In tumor cells, NRG1 expression was detected in the cytoplasm and HER3 expression in the cytoplasm and/or membrane of tumor cells. HER4 expression was also observed in the cytoplasm of tumor cells; however, a significant fraction of GC exhibited nuclear expression of HER4 (Fig. 2); Therefore, we recorded cytoplasmic and nuclear expression of HER4 separately. We evaluated both the extent (%) and the intensity of positive

tumor cells. The intensity of NRG1, HER3, and HER4 protein expression was classified into the following four categories according to the scoring system presented in a previous report [15]: 0, negative; 1+, weak positive; 2+, moderate positive; 3+, strong positive. For statistical analysis, cases with the immunostaining intensity of 2+ or 3+ in 10% or more tumor cells were defined as positive or overexpression of NRG1 and its receptors.

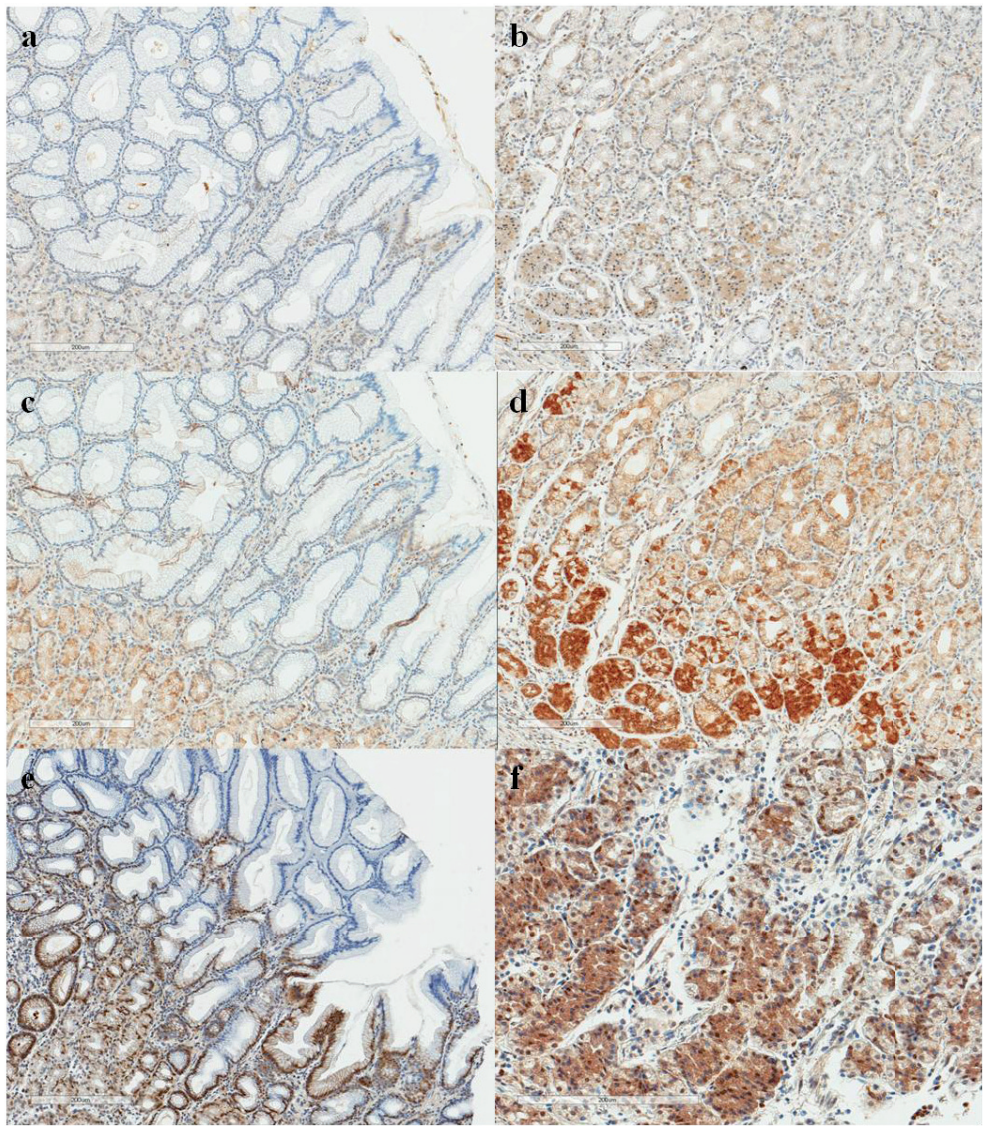

Figure 1. Representative images of NRG1, HER3, and HER4 protein expression in non-neoplastic gastric mucosa. **a** NRG1 in foveolar epithelium. **b** NRG1 in deep gland. **c** HER3 in foveolar epithelium. **d** HER3 in deep gland. **e** HER4 in foveolar epithelium. **f** HER4 in deep gland.

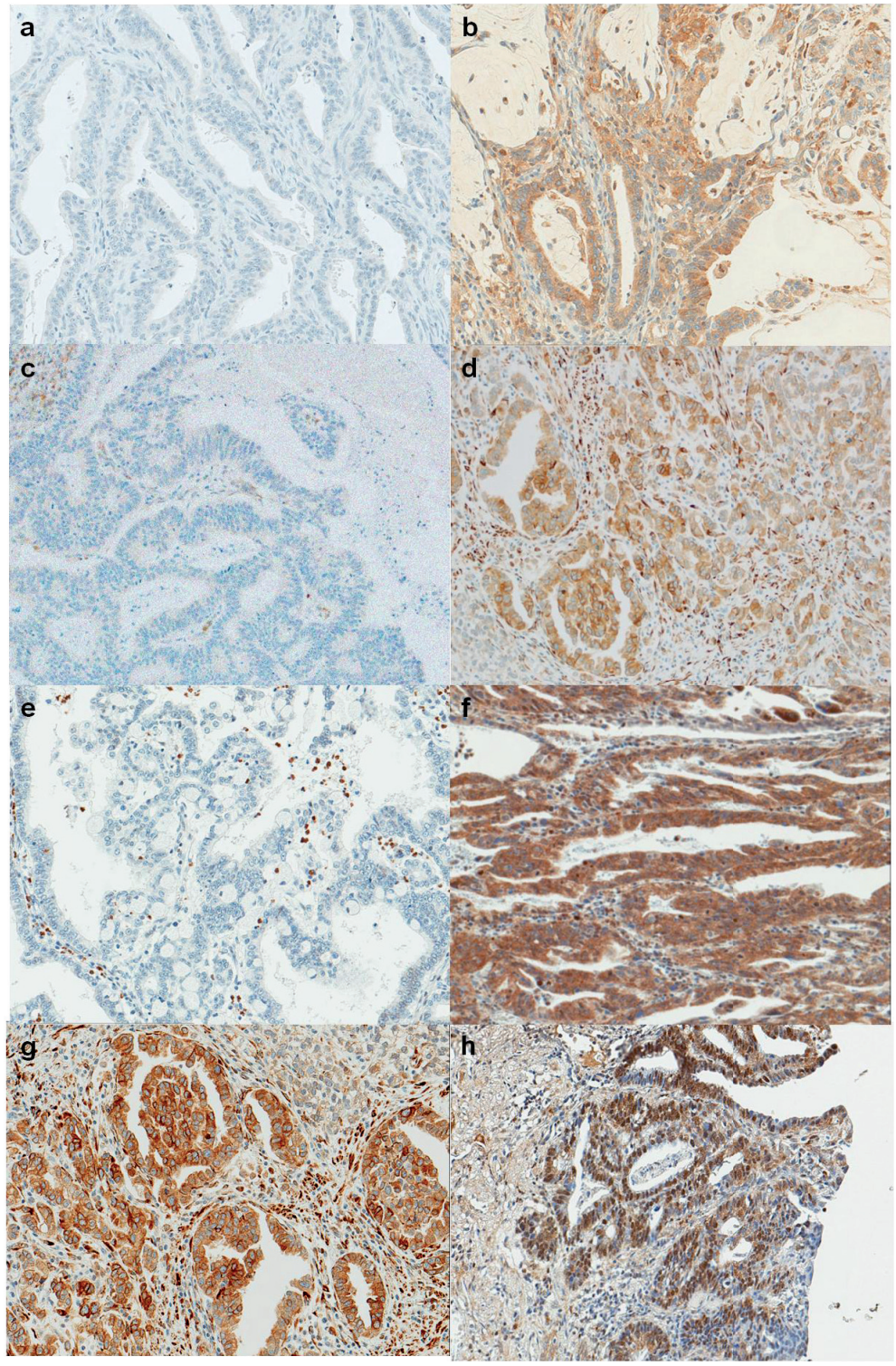
a
b
c
d
e
f
g
h

Figure 2. Representative images of NRG1, HER3, and HER4 protein expression in GC specimens. **a** NRG1 negative. **b** NRG1 positive. **c** HER3 negative. **d** HER3 cytoplasmic positive. **e** HER4 negative. **f** HER4 cytoplasmic positive. **g** HER3 membranous positive. **h** HER4 nuclear positive.

NRG1 analysis by dual-color fluorescence *in situ* hybridization

We performed FISH analysis to evaluate *NRG1* GCN. Of the 502 cases, 388 were interpretable by FISH analysis. Samples that were negative for tumor cells or without FISH signals were excluded. *NRG1* gene status was evaluated by dual-color FISH assay according to the manufacturer's instructions [23]. TMA slides (2 mm in thickness) were incubated with a NRG1 probe (Macrogen Inc., Seoul, Korea) and centromeric enumeration probe 8 (CEP8, Macrogen Inc., Seoul, Korea) with pepsin at 37℃ for 30 min. After being placed in HYBrite solution (Abbott Laboratories, Abbott Park, IL, USA) at 74℃, slides were counterstained with DAPI (Macrogen, Inc., Seoul, Korea). FISH analysis was evaluated without prior knowledge of clinicopathologic information. Entire cores were scanned and signals in 20 non-overlapping tumor nuclei counted in each core. If clusters were observed, small and large clusters were considered as 6 and 12 signals, respectively. *NRG1* amplification was defined as anNRG1/CEP8 ratio of ≥ 2.0. In addition to *NRG1* amplification, increased *NRG1* GCN signals were also observed. Since there are no standardized guidelines for evaluation of *NRG1* gene status, we used a cutoff value adapted from a previous study on EGFR in GC [24]; hence, *NRG1* GCN gain was defined as the copy number of NRG1 per nucleus of ≥ 2.5 (Fig. 3).

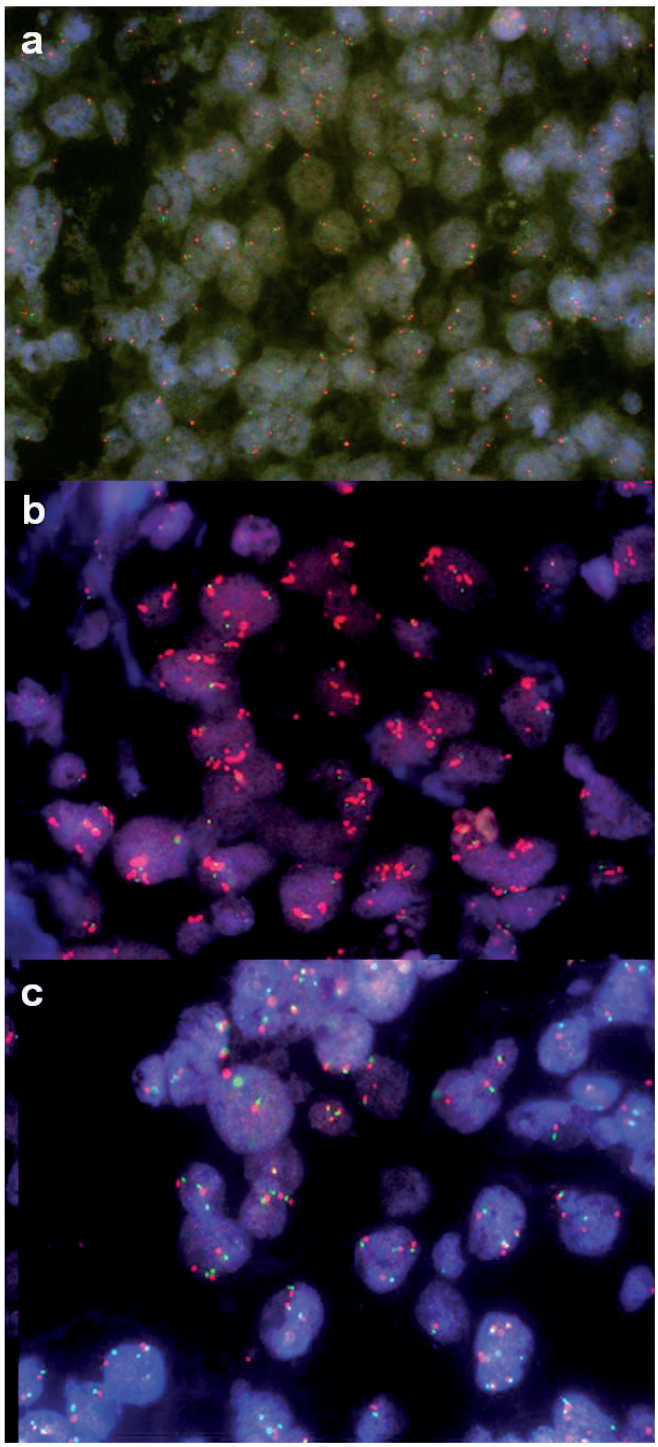

Figure 3. Representative images of the *NRG1* FISH assay in GC specimens. **a** *NRG1* GCN no gain. **b** *NRG1*amplification. **c** *NRG1* GCN gain.

Evaluation of HER2 status

HER2 status was determined according to the results of IHC and silver ISH (SISH), as described previously [25]. Briefly, HER2 protein expression was evaluated according to the DAKO guideline for scoring HercepTestTM in GC. *HER2* gene status was evaluated using a Ventana BenchMark XT device (Ventana Medical Systems). INFORM HER2 DNA and INFORM Chromosome 17 (CEP17) were used for automatic SISH staining. HER2 positivity was indicated when cancer cells had IHC scores of 2+ or 3+ in addition to *HER2* gene amplification based on SISH.

Microsatellite instability status

Tissue sections were obtained from formalin-fixed paraffin embedded blocks, and both tumor and normal areas were microdissected. After deparaffinization with incubation at 70℃ for 10 min, DNA was extracted using a chelating ion-exchange resin (Instagene matrix; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. MSI analysis was performed using an ABI 3731 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) with five microsatellite markers (BAT-26, BAT-25, D5S346, D17S250, and D2S123). MSI status was determined into MSI-high (two or more unstable markers), MSI-low (one unstable marker), or microsatellite stable (MSS, no unstable marker) [25].

Epstein-Barr virus *in situ* hybridization

EBV ISH using a fluorescein-conjugated EBER oligonucleotide probe (INFORM EBV-encoded RNA probe, Ventana Medical Systems) was performed to determine the EBV status of tumor samples. The cases with cancer cells positive for nuclear EBER were considered EBV-positive GC.

Statistical analysis

SPSS 21.0 (IBM Corp., Armonk, NY) was used for statistical analyses. Correlations between NRG1 or HER expression results and clinicopathologic variables were examined using Pearson's chi-square and Fisher's exact tests. The significance of associations with patient outcome was analyzed using Kaplan-Meier survival curves and compared using log rank tests. Univariate and multivariate analyses were performed for significant prognostic factors using Cox regression survival analysis. The concordance of NRG1 assessment by IHC and FISH was determined using a Spearman's rank correlation test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Clinicopathological characteristics of patients

The clinicopathological characteristics of 502 patients enrolled in this study are summarized in Table 1. The median age was 62 years (range 25–89 years); 332 (66.1%) were male and 170 (33.9%) female. At the time of surgical treatment, pTNM stages were distributed as follows: 256 (51.0%) cases were at stage I, 78 (15.5%) at stage II, 144 (28.7%) at stage III, and 24 (4.8%) at stage IV. By the Lauren classification, intestinal, diffuse, and mixed type tumors accounted for 217 (43.2%), 240 (47.8%), and 45 (9.0%) cases, respectively. Of the 502 cases, 239 (47.6%) had lymph node metastasis. MSI status was evaluated in 489 cases, and 40 (8.2%) cases were in the MSI-high group. EBV results were available from 501 GCs, among which EBV positivity was observed in 50 (10.0%) cases. NRG1 overexpression was detected in 141 (28.1%) cases. HER3 cytoplasmic overexpression was present in 157 (31.3%) cases, including 13 (2.6%) with membrane staining. Cytoplasmic HER4 expression was observed in 277 (55.2%) cases.

Table 1. Clinicopathological characteristics of patients

Characteristics	n (502)
Age (years), median (range)	62 (25-89)
Sex, male/female	332/170
Tumor size (cm) ≤3/>3	158/344
Tumor location	
Upper third	80
Middle third	156
Lower third	252
Entire	14
Lauren's classification	
Intestinal	217
Diffuse	240
Mixed	45
Ming classification, expanding/infiltrative	185/317
Vascular invasion, absent/present	445/57
Lymphatic invasion, absent/present	256/246
Neural invasion. absent/present	330/172
Depth of invasion, pT1,2/pT3,4	295/207
Lymph node metastasis, N0/N+	263/239
pTNM stage, I/II/III/IV	256/78/155/24
Tumor multiplicity, no/yes	471/31

Clinicopathologic significance of NRG1, HER3, and HER4 expression

The results of analyses of correlations between clinicopathologic variables are presented in Table 2, along with the expression status of NRG1, HER3, and HER4. NRG1 overexpression was more frequently identified in GC with unfavorable clinicopathologic features, including larger tumor size ($P < 0.001$), infiltrative tumor border ($P = 0.002$), vascular invasion ($P = 0.012$), lymphatic invasion ($P < 0.001$), neural invasion ($P < 0.001$), advanced pT stage ($P < 0.001$), lymph node metastasis ($P < 0.001$), and advanced pTNM stage ($P < 0.001$). However, there was no significant correlation between NRG1 positivity by IHC and age, sex, location, or Lauren classification ($P = 0.338$, 0.793, 0.244, and 0.150, respectively). Among 502 cases, HER3 cytoplasmic overexpression correlated strongly with older age ($P < 0.001$) and an expanding tumor border ($P = 0.007$). HER3 cytoplasmic overexpression was also more frequently detected in intestinal or mixed type GC than in diffuse type GC ($P < 0.001$) and tended to be detected in tumors located in the lower third of the stomach ($P = 0.021$). HER4 cytoplasmic expression did not show any significant association with clinicopathologic characteristics except age ($P = 0.011$) and histologic type by the Lauren classification ($P = 0.007$). HER2, MSI, and EBV status exhibited no significant correlations with NRG1, HER3, or HER4 expression (all $P > 0.05$) other than a correlation between HER2 and HER3 cytoplasmic expression ($P = 0.022$).

able 2. The correlation between clinicopathologic parameters and expression status of NRG1, HER3, and HER4

Characteristics	NRG1 (%)			HER3 (cytoplasmic) (%)			HER4 (cytoplasmic) (%)		
	Negative	**Positive**	***P***	**Negative**	**Positive**	***P***	**Negative**	**Positive**	***P***
otal	361 (71.9)	141 (28.1)		345 (68.7)	157 (31.3)		225 (44.8)	277 (55.2)	
Age (years)			0.338			<0.001			0.011
≤60	157 (69.8)	68 (30.2)		173 (76.9)	52 (23.1)		115 (51.1)	110 (48.9)	
>60	204 (73.6)	73 (26.4)		172 (62.1)	105 (37.9)		110 (39.7)	167 (60.3)	
Sex			0.793			0.097			0.139
Male	240 (72.3)	92 (27.7)		220 (66.3)	112 (33.7)		141 (42.5)	191 (57.5)	
Female	121 (71.2)	49 (28.8)		125 (73.5)	45 (26.5)		84 (49.4)	86 (50.6)	
umor size			<0.001			0.932			0.726
≤3cm	131 (82.9)	27 (17.1)		109 (69.0)	49 (31.0)		69 (43.7)	89 (56.3)	
>3cm	230 (66.9)	114 (33.1)		236 (68.6)	108 (31.4)		156 (45.3)	188 (54.7)	
ocation			0.244			0.021			0.139
Upper third	51 (63.8)	29 (36.3)		59 (73.8)	21 (26.3)		33 (41.3)	47 (58.8)	
Middle third	119 (76.3)	37 (23.7)		118 (75.6)	38 (24.4)		82 (52.6)	74 (47.4)	

Lower third	181 (71.8)	71 (28.2)		157 (62.3)	95 (37.7)		104 (41.3)	148 (58.7)	
Entire	10 (71.4)	4 (28.6)		11 (78.6)	3 (21.4)		6 (42.9)	8 (57.1)	
Lauren classification			0.150			<0.001			0.007
Intestinal type	164 (75.6)	53 (24.4)		122 (56.2)	95 (43.8)		82 (37.8)	135 (62.2)	
Diffuse type	169 (70.4)	71 (29.6)		196 (81.7)	44 (18.3)		125 (52.1)	115 (47.9)	
Mixed type	28 (62.2)	17 (37.8)		27 (60.0)	18 (40.0)		18 (40.0)	27 (60.0)	
Ming classification			0.002			0.005			0.141
Expanding	148 (80.0)	37 (20.0)		113 (61.1)	72 (38.9)		75 (40.5)	110 (59.5)	
Infiltrative	213 (67.2)	104 (32.8)		232 (73.2)	85 (26.8)		150 (47.3)	167 (52.7)	
Vascular invasion			0.012			0.579			0.661
Absent	328 (73.7)	117 (26.3)		304 (68.3)	141 (31.7)		201 (45.2)	244 (54.8)	
Present	33 (57.9)	24 (42.1)		41 (71.9)	16 (28.1)		24 (42.1)	33 (57.9)	
Lymphatic invasion			<0.001			0.243			0.193
Absent	208 (81.3)	48 (18.8)		182 (71.1)	74 (28.9)		122 (47.7)	134 (52.3)	
Present	153 (62.2)	93 (37.8)		163 (66.3)	83 (33.7)		103 (41.9)	143 (58.1)	
Neural invasion			<0.001			0.571			0.086

Absent	264 (80.0)	66 (20.0)		224 (67.9)	106 (32.1)		157 (47.6)	173 (52.4)	
Present	97 (56.4)	75 (43.6)		121 (70.3)	51 (29.7)		68 (39.5)	104 (60.5)	
Depth of invasion (pT)			<0.001			0.221			0.156
T1-T2	239 (81.0)	56 (19.0)		209 (70.8)	86 (29.2)		140 (47.5)	155 (52.5)	
T3-T4	122 (58.9)	85 (41.1)		136 (65.7)	71 (34.3)		85 (41.1)	122 (58.9)	
Lymph node metastasis			<0.001			0.809			0.459
N0	213 (81.0)	50 (19.0)		182 (69.2)	81 (30.8)		122 (46.4)	141 (53.6)	
N(+)	148 (61.9)	91 (38.1)		163 (68.2)	76 (31.8)		103 (43.1)	136 (56.9)	
pTNM stage			<0.001			0.604			0.607
I-II	262 (78.4)	72 (21.6)		227 (68.0)	107 (32.0)		147 (44.0)	187 (56.0)	
III-IV	99 (58.9)	69 (41.1)		118 (70.2)	50 (29.8)		78 (46.4)	90 (53.6)	
Tumor multiplicity			0.264			0.186			0.739
No	336 (71.3)	135 (28.7)		327 (69.4)	144 (30.6)		212 (45.0)	259 (55.0)	
Yes	25 (80.6)	6 (19.4)		18 (58.1)	13 (41.9)		13(41.9)	18 (58.1)	
HER2 status			0.992			0.022			0.186
Negative	343 (71.9)	134 (28.1)		333 (69.8)	144 (30.2)		217 (45.5)	260 (54.5)	

Positive	18 (72.0)	7 (28.0)		12 (48.0)	13 (52.0)		8 (32.0)	17 (68.0)	
MSI status (n = 489)			0.336			0.215			0.762
MSS/MSI-L	324 (72.2)	125 (27.8)		312 (69.5)	137 (30.5)		202 (45.0)	247 (55.0)	
MSI-H	26 (65.0)	14 (35.0)		24 (60.0)	16 (40.0)		17 (42.5)	23 (57.5)	
EBV status (n = 501)			0.332			0.454			0.192
Negative	327 (72.5)	124 (27.5)		312 (69.2)	139 (30.8)		206 (45.7)	245 (54.3)	
Positive	33 (66.0)	17 (34.0)		32 (64.0)	18 (36.0)		18 (36.0)	32 (64.0)	

Survival analysis

For survival analysis, 501 patients were followed up for 1–109 months, with a median follow-up period of 67 months. The remaining single case was lost to follow-up after surgery. At the time of analysis, 118 (23.6%) patients had tumor recurrence and 110 (22.0%) suffered disease-related death. Kaplan-Meier survival analysis revealed that patients with GC overexpressing NRG1 had significantly worse DFS and DSS compared to the NRG1 negative group (both $P < 0.001$); however, there was no difference in DFS or DSS associated with HER3 or HER4 cytoplasmic overexpression (both $P > 0.05$; Fig.4). After exclusion of two patients with ≤1 months of follow up, we also performed Kaplan-Meier survival analysis, and similar results were observed (Fig. 5). Univariate analysis indicated that NRG1 expression and established prognostic pathologic factors, including tumor size, non-intestinal histology, tumor border, vascular invasion, lymphatic invasion, neural invasion, and pathologic stage, were significantly associated with DFS and DSS. By multivariate analysis, NRG1 overexpression was identified as an unfavorable prognostic factor for DFS (hazard ratio 1.455; 95% confidence interval 1.009–2.100; $P = 0.045$) and DSS (hazard ratio 1.490; 95% confidence interval 1.019–2.177; $P = 0.040$). Vascular invasion, lymphatic invasion, and pTNM stage were independent prognostic factors for both DFS and DSS. Neural invasion was also independently associated with DSS (Table 3).

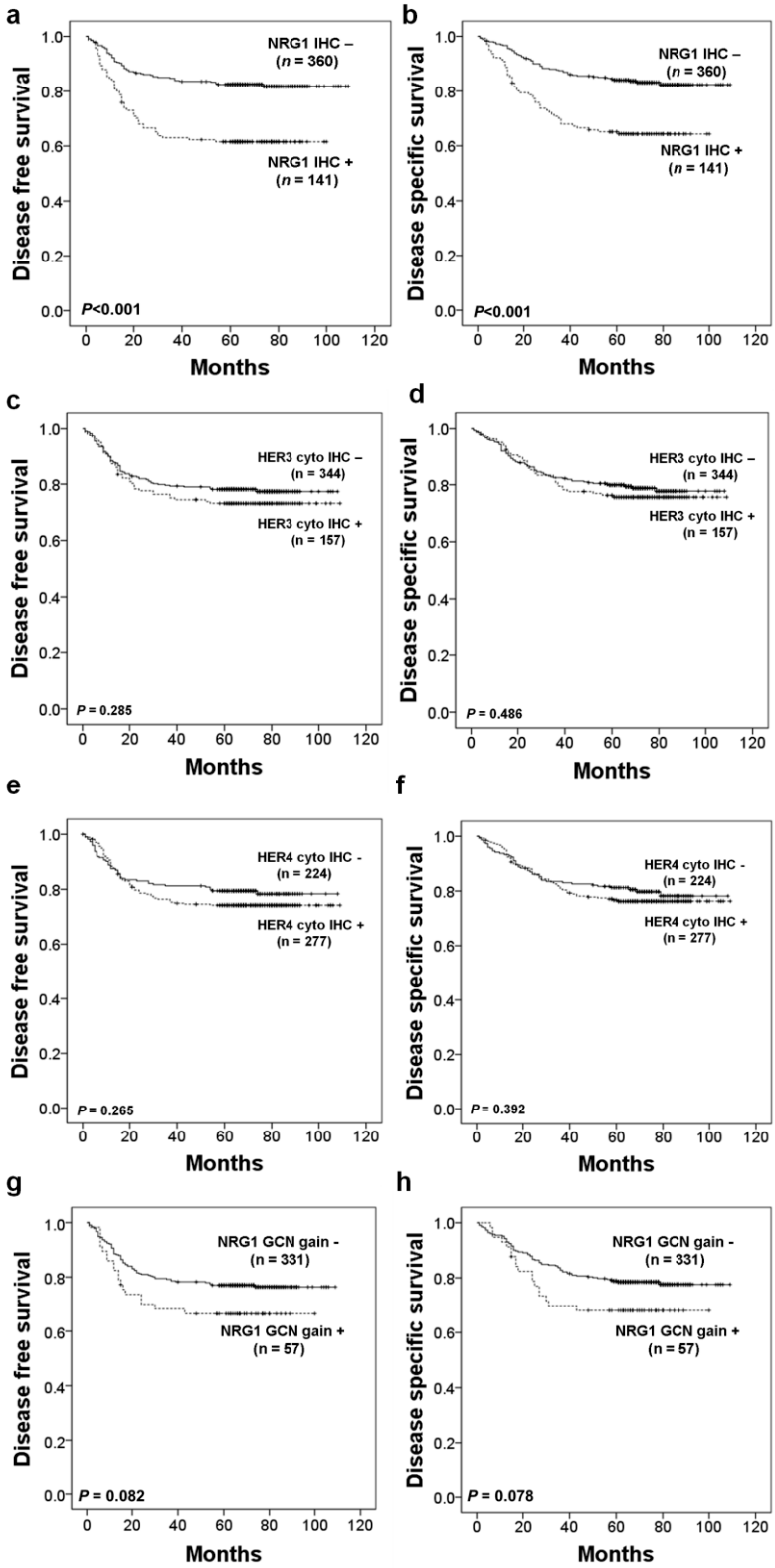
a
Disease free survival
NRG1 IHC – (n = 360)
NRG1 IHC + (n = 141)
P<0.001
Months
b
Disease specific survival
NRG1 IHC – (n = 360)
NRG1 IHC + (n = 141)
P<0.001
Months
c
Disease free survival
HER3 cyto IHC – (n = 344)
HER3 cyto IHC + (n = 157)
P = 0.285
Months
d
Disease specific survival
HER3 cyto IHC – (n = 344)
HER3 cyto IHC + (n = 157)
P = 0.486
Months
e
Disease free survival
HER4 cyto IHC - (n = 224)
HER4 cyto IHC + (n = 277)
P = 0.265
Months
f
Disease specific survival
HER4 cyto IHC - (n = 224)
HER4 cyto IHC + (n = 277)
P = 0.392
Months
g
Disease free survival
NRG1 GCN gain - (n = 331)
NRG1 GCN gain + (n = 57)
P = 0.082
Months
h
Disease specific survival
NRG1 GCN gain - (n = 331)
NRG1 GCN gain + (n = 57)
P = 0.078
Months

Figure 4. Kaplan-Meier survival estimates according to NRG1, HER3 and HER4 protein expression, and *NRG1* gene copy number status. Disease free survival and disease specific survival according to **a**, **b** NRG1, **c**, **d** HER3 cytoplasmic, **e**, **f** HER4 cytoplasmic expression, and **g**, **h** *NRG1* GCN status.

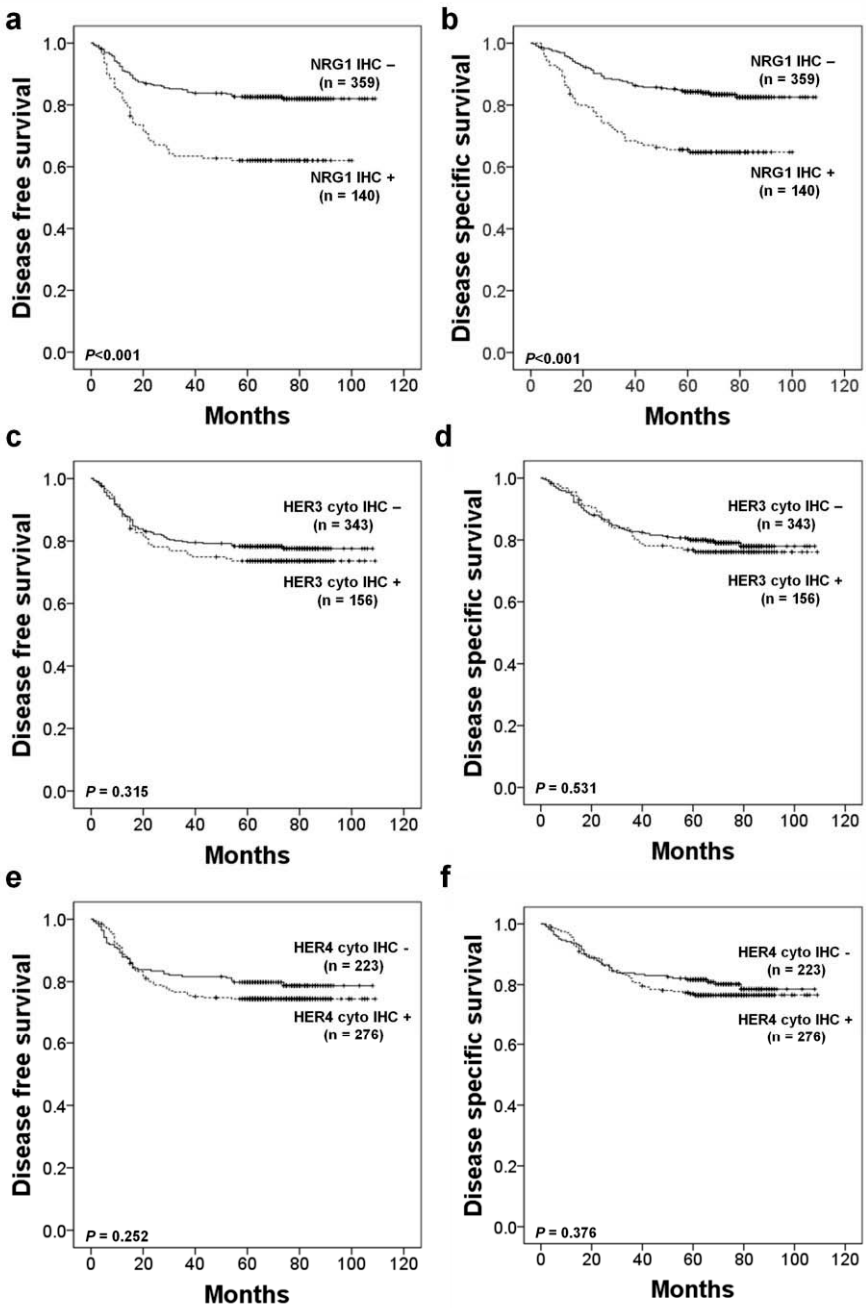
a
Disease free survival
NRG1 IHC –
(n = 359)
NRG1 IHC +
(n = 140)
P<0.001
Months
b
Disease specific survival
NRG1 IHC –
(n = 359)
NRG1 IHC +
(n = 140)
P<0.001
Months
c
Disease free survival
HER3 cyto IHC –
(n = 343)
HER3 cyto IHC +
(n = 156)
P = 0.315
Months
d
Disease specific survival
HER3 cyto IHC –
(n = 343)
HER3 cyto IHC +
(n = 156)
P = 0.531
Months
e
Disease free survival
HER4 cyto IHC -
(n = 223)
HER4 cyto IHC +
(n = 276)
P = 0.252
Months
f
Disease specific survival
HER4 cyto IHC -
(n = 223)
HER4 cyto IHC +
(n = 276)
P = 0.376
Months

Figure 5. Kaplan-Meier survival estimates according to NRG1, HER3 and HER4 protein expression (excluding cases with ≤1 months of follow-up). Disease free survival and disease specific survival according to **a**, **b** NRG1, **c**, **d** HER3 cytoplasmic, and **e**, **f** HER4 cytoplasmic expression.

Table 3. Univariate and multivariate analysis for disease free survival and disease specific survival in gastric cancer

Variables	Category	Disease free survival		Disease specific survival	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Univariate analysis					
Age (years)	>60 vs. ≤60	1.154 (0.801-1.662)	0.443	1.245 (0.851-1.821)	0.258
Tumor size	>3cm vs. ≤3cm	10.163 (4.469-23.110)	<0.001	11.309 (4.610-27.741)	<0.001
Lauren classification	Non-intestinal vs. intestinal	2.228 (1.485-3.344)	<0.001	2.115 (1.396-3.204)	<0.001
Ming classification	Infiltrative vs. expanding	3.217 (1.988-5.205)	<0.001	3.400 (2.051-5.636)	<0.001
Vascular invasion	Present vs. absent	6.078 (4.137-8.932)	<0.001	6.364 (4.279-9.464)	<0.001
Lymphatic invasion	Present vs. absent	8.938 (5.197-15.372)	<0.001	9.541 (5.345-17.031)	<0.001
Neural invasion	Present vs. absent	7.900 (5.187-12.030)	<0.001	8.705 (5.566-13.614)	<0.001
pTNM stage	III,IV vs. I,II	24.361 (13.658-43.452)	<0.001	23.829 (13.063-43.470)	<0.001
MSI status	MSI_H vs. MSS/MSI-L	0.588 (0.259-1.338)	0.205	0.626 (0.275-1.425)	0.264
NRG1	Positive vs. negative	2.458 (1.710-3.532)	<0.001	2.450 (1.683-3.567)	<0.001

HER3 (cytoplasm)	Positive vs. negative	1.227 (0.842-1.788)	0.288	1.150 (0.776-1.703)	0.487
HER3 (membrane)	Positive vs. negative	2.595 (1.141-5.902)	0.023	2.657 (1.166-6.052)	0.020
HER4 (cytoplasm)	Positive vs. negative	1.232 (0.852-1.781)	0.268	1.180 (0.807-1.726)	0.393
HER4 (nucleus)	Positive vs. negative	0.305 (0.164-0.567)	<0.001	0.263 (0.133-0.520)	<0.001
Multivariate analysis					
Tumor size	>3cm vs. ≤3cm	2.171 (0.901-5.228)	0.084	2.395 (0.924-6.208)	0.072
Lauren classification	Non-intestinal vs. intestinal	0.810 (0.531-1.236)	0.328	0.775 (0.503-1.194)	0.248
Ming classification	Infiltrative vs. expanding	0.894 (0.528-1.514)	0.677	0.975 (0.562-1.691)	0.928
Vascular invasion	Present vs. absent	1.785 (1.203-2.648)	0.004	1.846 (1.228-2.776)	0.003
Lymphatic invasion	Present vs. absent	1.911 (1.044-3.499)	0.036	2.086 (1.110-3.919)	0.022
Neural invasion	Present vs. absent	1.450 (0.890-2.363)	0.136	1.655 (1.008-2.717)	0.046
pTNM stage	III,IV vs. I,II	14.008 (7.312-26.836)	<0.001	9.896 (4.875-20.086)	<0.001
NRG1	Positive vs. negative	1.455 (1.009-2.100)	0.045	1.490 (1.019-2.177)	0.040
HER3(membrane)	Positive vs. negative	1.835 (0.798-4.218)	0.153	1.744 (0.757-4.016)	0.191
HER4 (nucleus)	Positive vs. negative	1.088 (0.561-2.111)	0.803	0.901 (0.438-1.854)	0.778

Evaluation of *NRG1* GCN by FISH

The median *NRG1*/CEP8 ratio was 1.03 (range 0.57–5.72). Among the available 388 cases, *NRG1* GCN gain was detected in 57 (14.7%), including 2 (0.5%) cases of amplification. When *NRG1* GCN status was compared with NRG1 protein expression, *NRG1* GCN gain was significantly associated with NRG1 protein expression ($P < 0.001$; kappa = 0.459; Table 4). However, the two cases withNRG1amplification were negative for NRG1 by IHC analysis, and *NRG1* GCN gain was not observed in the majority of NRG1 IHC-positive cases (65/113, 57.5%). *NRG1* GCN gain was significantly associated with diffuse or mixed type by the Lauren classification ($P = 0.001$), lymphatic invasion ($P = 0.013$), and lymph node metastasis ($P = 0.013$; Table 5). By Kaplan-Meier analysis, patients with *NRG1* GCN gain had shorter DFS and DSS with borderline statistical significance ($P = 0.082$ and $P = 0.078$, respectively; Fig.2), but Cox regression analysis indicated that *NRG1* GCN gain was not an independent prognostic factor ($P > 0.05$).

Table 4. Correlation between NRG1 immunohistochemistry and gene copy number status.

	NRG1 IHC		*P*	*κ*
	Negative	Positive		
***NRG1* GCN**				
GCN non-gain	266 (80.4%)	65 (19.6%)	<0.001	0.459
GCN gain	9 (15.8%)	48 (84.2%)		

Table 5. Clinicopathological implications of *NRG1* gene copy number gain

Characteristics	*NRG1* GCN gain (%)		
	Negative	Positive	*P*
Total	331 (85.3)	57 (17.4)	
Age (years)			0.746
≤60	147 (86.0)	24 (14.0)	
>60	184 (84.8)	33 (15.2)	
Sex			0.417
Male	220 (84.3)	41 (15.7)	
Female	111 (87.4)	16 (12.6)	
Tumor size			0.092
≤3cm	100 (90.2)	11 (9.9)	
>3cm	231 (83.4)	46 (16.6)	
Location			0.665
Upper third	55 (85.9)	9 (14.1)	
Middle third	101 (87.1)	15 (12.9)	
Lower third	166 (84.7)	30 (15.3)	
Entire	9 (75.0)	3 (25.0)	
Lauren classification			0.001
Intestinal type	151 (89.3)	18 (10.7)	
Diffuse type	154 (85.6)	26 (14.4)	
Mixed type	26 (66.7)	13 (33.3)	
Ming classification			0.768
Expanding	117 (86.0)	19 (14.0)	
Infiltrative	214 (84.9)	38 (15.1)	
Vascular invasion			0.982
Absent	290 (85.3)	50 (14.7)	
Present	41 (85.4)	7 (14.6)	
Lymphatic invasion			0.013
Absent	169 (89.9)	19 (10.1)	
Present	162 (81.0)	38 (19.0)	
Neural invasion			0.305
Absent	215 (86.7)	33 (13.3)	
Present	116 (82.9)	24 (17.1)	
Depth of invasion (pT)			0.263
T1-T2	189 (87.1)	28 (12.9)	

T3-T4	142 (83.0)	29 (17.0)	
Lymph node metastasis			0.013
N0	169 (89.9)	19 (10.1)	
N(+)	162 (81.0)	38 (19.0)	
pTNM stage			0.086
I-II	219 (87.6)	31 (12.4)	
III-IV	112 (81.2)	26 (18.8)	
HER2 status			0.224
Negative	310 (84.7)	56 (15.3)	
Positive	21 (95.5)	1 (4.5)	
MSI status (n = 489)			0.408
MSS/MSI-L	304 (85.9)	50 (14.1)	
MSI-H	23 (79.3)	6 (20.7)	
EBV status (n = 501)			0.099
Negative	302 (86.3)	48 (13.7)	
Positive	29 (76.3)	9 (23.7)	

Correlation between HER3 membranous expression and clinicopathologic factors

Positive expression of HER3 was predominantly observed in the cytoplasm, and 13 of 502 cases (2.6%) also showed HER3 membranous expression (Fig. 2). GC cases with HER3 membranous expression correlated with lymphatic invasion (P = 0.009), lymph node metastasis (P = 0.032), and mixed type according to the Lauren classification (P = 0.002), but did not correlate with other clinicopathologic factors including MSI and EBV status (all $P > 0.05$; Table 6). GC patients with HER3 membranous expression had an unfavorable outcome for DFS (P = 0.018) and DSS (P = 0.015) by survival analysis (Fig. 6 and Fig. 7). However, it was not an independent prognostic factor by multivariate analysis for DFS (P = 0.153) and DSS (P = 0.191; Table 3).

Table 6. Clinicopathological implications of HER3 membranous and HER4 nuclear expression

Characteristics	HER3, membranous (%)			HER4, nuclear (%)		
	Negative	Positive	*P*	Negative	Positive	*P*
Total	489 (97.4)	13 (2.6)		387 (77.1)	115 (22.9)	
Age (years)			0.640			0.162
≤60	220 (97.8)	5 (2.2)		180 (80.0)	45 (20.0)	
>60	269 (97.1)	8 (2.9)		207 (74.7)	70 (25.3)	
Sex			0.770			0.267
Male	324 (97.6)	8 (2.4)		251 (75.6)	81 (24.4)	
Female	165 (97.1)	5 (2.9)		136 (80.0)	34 (20.0)	
Tumor size			0.072			0.007
≤3cm	157 (99.4)	1 (0.6)		110 (69.6)	48 (30.4)	
>3cm	332 (96.5)	12 (3.5)		277 (80.5)	67 (19.5)	
Location			0.610			0.103
Upper third	243 (96.4)	9 (3.6)		186 (73.8)	66 (26.2)	
Middle third	154 (98.7)	2 (1.3)		120 (76.9)	36 (23.1)	
Lower third	78 (97.5)	2 (2.5)		68 (85.0)	12 (15.0)	
Entire	14 (100)	0 (0)		13 (92.9)	1 (7.1)	
Lauren classification			0.002			0.052
Intestinal type	211 (97.2)	6 (2.8)		156 (71.9)	61 (28.1)	

Diffuse type	237 (98.8)	2 (1.3)		194 (80.8)	46 (19.2)	
Mixed type	41 (91.1)	5 (8.9)		37 (82.2)	8 (17.8)	
Ming classification			1.000			0.019
Expanding	180 (97.3)	5 (2.7)		132 (71.4)	53 (28.6)	
Infiltrative	309 (97.5)	8 (2.5)		255 (80.4)	62 (19.6)	
Vascular invasion			0.650			0.018
Absent	434 (97.5)	11 (2.5)		336 (75.5)	109 (24.5)	
Present	55 (96.5)	2 (3.5)		51 (89.5)	6 (10.5)	
Lymphatic invasion			0.009			0.001
Absent	254 (99.2)	2 (0.8)		182 (71.1)	74 (28.9)	
Present	235 (95.5)	11 (4.5)		205 (83.3)	41 (16.7)	
Neural invasion			0.146			<0.001
Absent	324 (98.2)	6 (1.8)		235 (71.2)	95 (28.8)	
Present	165 (95.9)	7 (4.1)		152 (88.4)	20 (11.6)	
Depth of invasion (pT)			0.132			<0.001
T1-T2	290 (98.3)	5 (1.7)		204 (69.2)	91 (30.8)	
T3-T4	199 (96.1)	8 (3.9)		183 (88.4)	24 (11.6)	
Lymph node metastasis			0.032			0.002
N0	260 (98.9)	3 (1.1)		188 (71.5)	75 (28.5)	
N(+)	229 (95.8)	10 (4.2)		199 (83.3)	40 (16.7)	

pTNM stage			0.139			0.001
I-II	328 (98.2)	6 (1.8)		232 (69.5)	102 (30.5)	
III-IV	161 (95.8)	7 (4.2)		155 (92.3)	13 (7.7)	
HER2 status			1.000			0.723
Negative	464 (97.3)	13 (2.7)		367 (76.9)	110 (23.1)	
Positive	25 (100)	0 (0.0)		20 (80.0)	5 (20.0)	
MSI status (n = 489)			0.288			0.020
MSS/MSI-L	438 (97.6)	11 (2.4)		344 (76.6)	105 (23.4)	
MSI-H	38 (95.0)	2 (5.0)		37 (92.5)	3 (7.5)	
EBV status (n = 501)			1.000			0.109
Negative	439 (97.3)	12 (2.7)		352 (78.0)	99 (22.0)	
Positive	49 (98.0)	1 (2.0)		34 (68.0)	16 (32.0)	

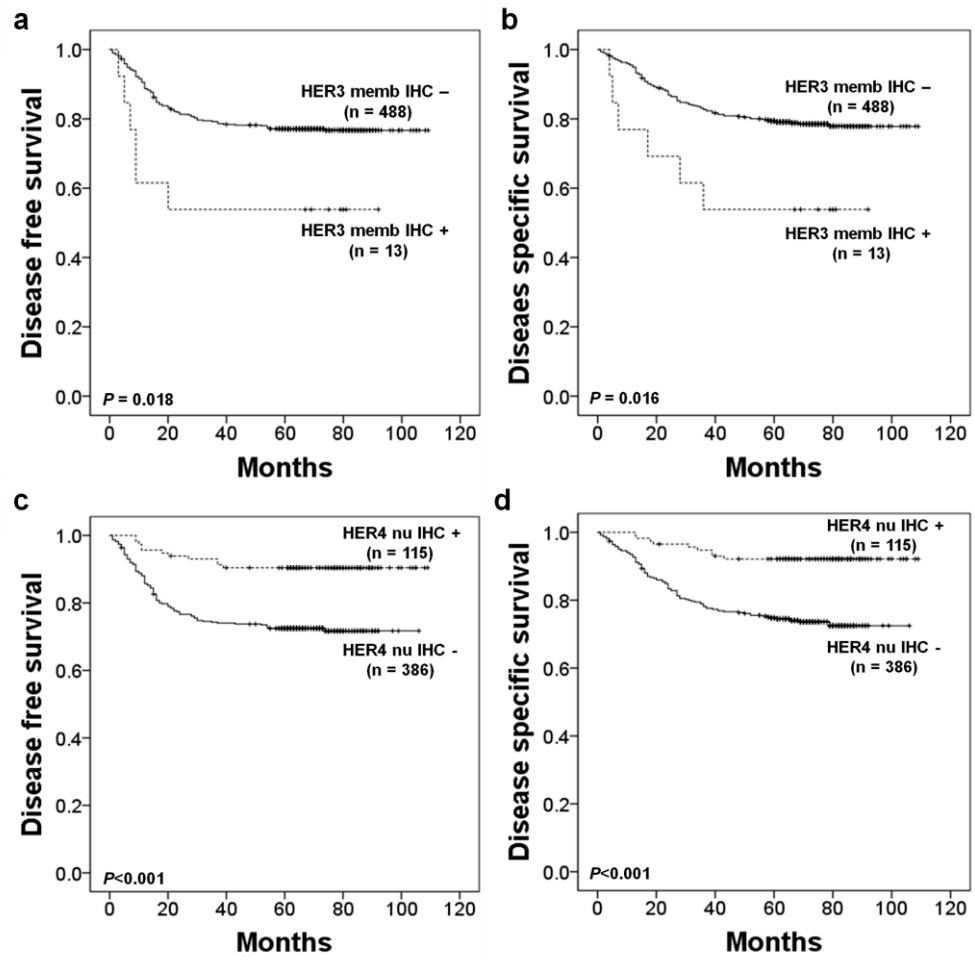

Figure 6. Survival curves according to **a, b** HER3 membranous and **c, d** HER4 nuclear expression.

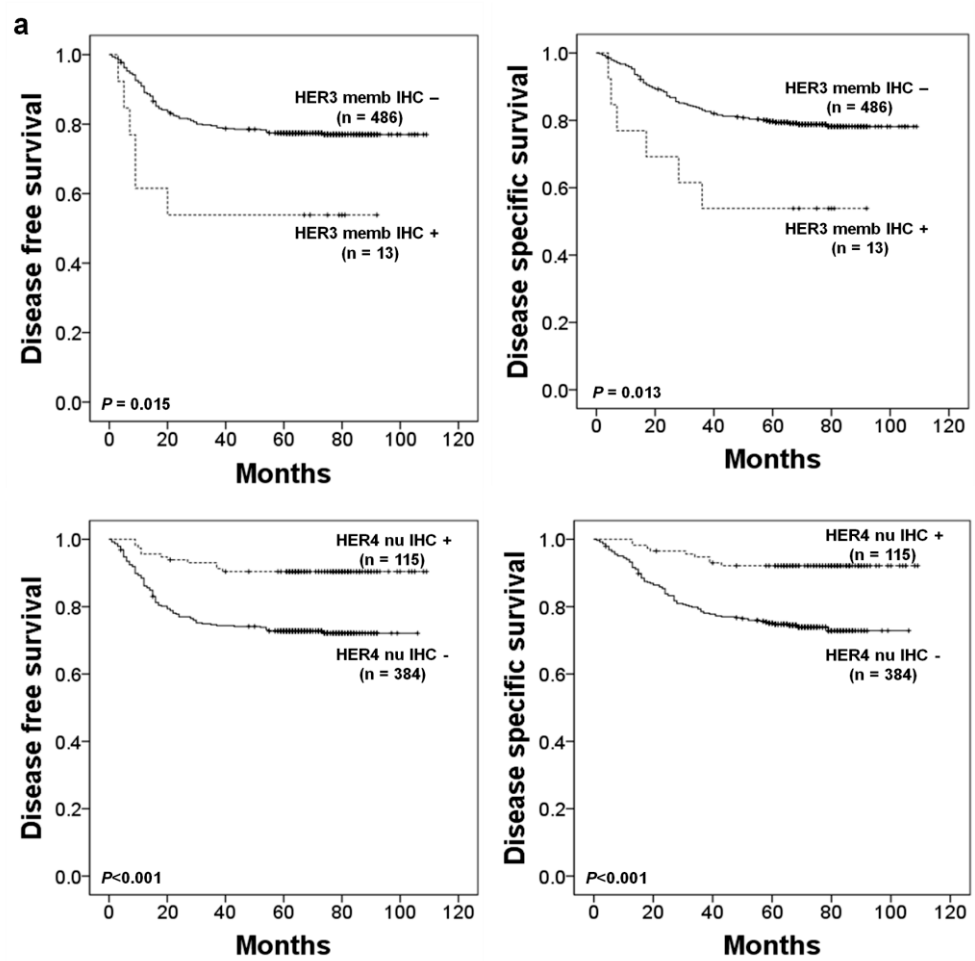

Figure 7. Survival curves according to **a, b** HER3 membranous and **c, d** HER4 nuclear expression (excluding cases with ≤1 months of follow-up).

Clinicopathologic significance of HER4 nuclear expression

We next evaluated the clinical significance of HER4 nuclear expression in GC. HER4 nuclear expression was observed in 115 (22.9%) of 502 GC cases (Fig. 2). HER4 nuclear expression was significantly associated with less aggressive clinicopathologic features, such as smaller tumor size, expanding tumor border, absence of lymphovascular and neural invasion, and early pathologic stage (all $P < 0.05$). HER4 nuclear expression was also associated with intestinal type GC with borderline statistical significance ($P = 0.052$; Table 6). In survival analysis, the HER4 nuclear expression group had superior DFS and DSS (both $P < 0.001$; Fig. 6 and Fig. 7); however, in a multivariate hazard model, it no longer exhibited prognostic significance for either DFS or DSS ($P = 0.803$ and $P = 0.778$, respectively; Table 3).

Correlation of NRG1 expression status with that of its receptors

To investigate associations between NRG1 and its receptors, we evaluated the results of NRG1 IHC in comparison with those for HER3 and HER4. As shown in Table 7, there was a close association between NRG1 and HER3 cytoplasmic expression ($P = 0.034$) and between NRG1 and HER4 cytoplasmic expression ($P < 0.001$). However, HER3 membranous expression and HER4 nuclear expression were not correlated with NRG1 expression, contrary to its cytoplasmic expression ($P = 0.763$ and $P = 0.084$, respectively).

Table 7. Correlation between expression of NRG1, HER3, and HER4

	NRG1		
	Negative	Positive	*P*
HER3 (cytoplasm)			0.034
Negative	258 (74.8%)	87 (25.2%)	
Positive	103 (65.6%)	54 (34.4%)	
HER3 (membrane)			0.763
Negative	352 (72.0%)	137 (28.0%)	
Positive	9 (69.2%)	4 (30.8%)	
HER4 (cytoplasm)			<0.001
Negative	184 (81.8%)	41 (18.2%)	
Positive	177 (63.9%)	100 (36.1%)	
HER4 (nucleus)			0.084
Negative	271 (70.0%)	116 (30.0%)	
Positive	90 (78.3%)	25 (21.7%)	

NRG1 and HER3 expression in GC patients receiving trastuzumab combined chemotherapy

Next, we collected 14 HER2 positive GC patients who had chemotherapy combined with HER2 inhibitor, trastzumab, and performed immunohistochemical analysis of NRG1 and HER3. Based on the results of previous studies that NRG1/HER3 signaling was associated with development of resistance to HER2-targeted treatment [8-11], we inferred that NRG1/HER3 coexpression group showed worse prognosis in trastzumab treated GC patients. The results of immunohistochemical staining were presented in Table 8 and Fig. 8. Among a total of 14 cases, NRG1 and HER3 expression were observed in 3 (21.4%) and 5 (35.7%), respectively. The coexpression of NRG1 and HER3 was found in 2 (14.3%) cases. Interestingly, HER3 expression was detected on membrane and/or cytoplasm of cancer cells. We analyzed the survival difference according to expression status of NRG1 and HER3. Each NRG1 and HER3 positive group showed a tendency of shorter progression free survival (PFS), but the statistical significance was not reached (P = 0.582 and P = 0.215, respectively; Fig. 9). NRG1 and HER3 coexpressed GC group also showed a shorter PFS, in agreement with our hypothesis (P = 0.005, Fig. 9). More validated studies for prognostic features and chemotherapy responses according to the NRG1/HER3 status in larger cohort of GC are necessary.

Table 8. Clinicopathologic characteristics of HER2 positive and trastzumab treated GC cases and immunohistochemical staining of NRG1 and HER3

	Age/Sex	Metastasis	Lauren classification	NRG1	HER3	Cancer progression
Case 1	31/M	(-)	Diffuse	(-)	**(+)**	**(+)**
Case 2	62/F	(-)	Mixed	(-)	(-)	**(+)**
Case 3	73/M	(-)	Diffuse	(-)	(-)	**(+)**
Case 4	76/M	(-)	Intestinal	(-)	(-)	**(+)**
Case 5	47/M	(-)	Diffuse	(-)	(-)	(-)
Case 6	54/M	(-)	Intestinal	(-)	**(+)**	**(+)**
Case 7	60/M	(-)	Intestinal	**(+)**	(-)	(-)
Case 8	68/M	(+)	Intestinal	(-)	(-)	**(+)**
Case 9	62/M	(-)	Intestinal	(-)	(-)	(-)
Case 10	49/M	(-)	Intestinal	**(+)**	**(+)**	**(+)**
Case 11	58/F	(+)	Diffuse	(-)	(-)	**(+)**
Case 12	46/M	(-)	Intestinal	(-)	**(+)**	**(+)**
Case 13	63/M	(+)	Intestinal	(-)	(-)	(-)
Case 14	53/M	(-)	Diffuse	**(+)**	**(+)**	**(+)**

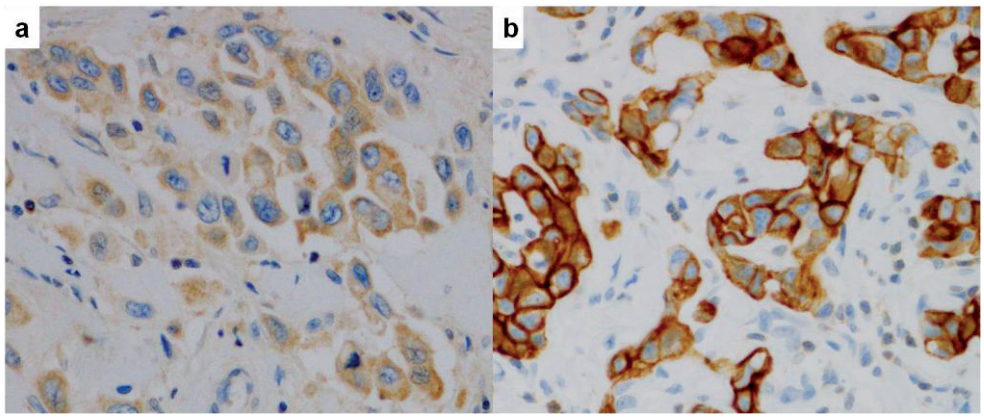

Figure 8. NRG1 and HER3 expression in HER2 positive GC patients who had chemotherapy combined with HER2 inhibitor, trastzumab. **a** NRG1. **b** HER3.

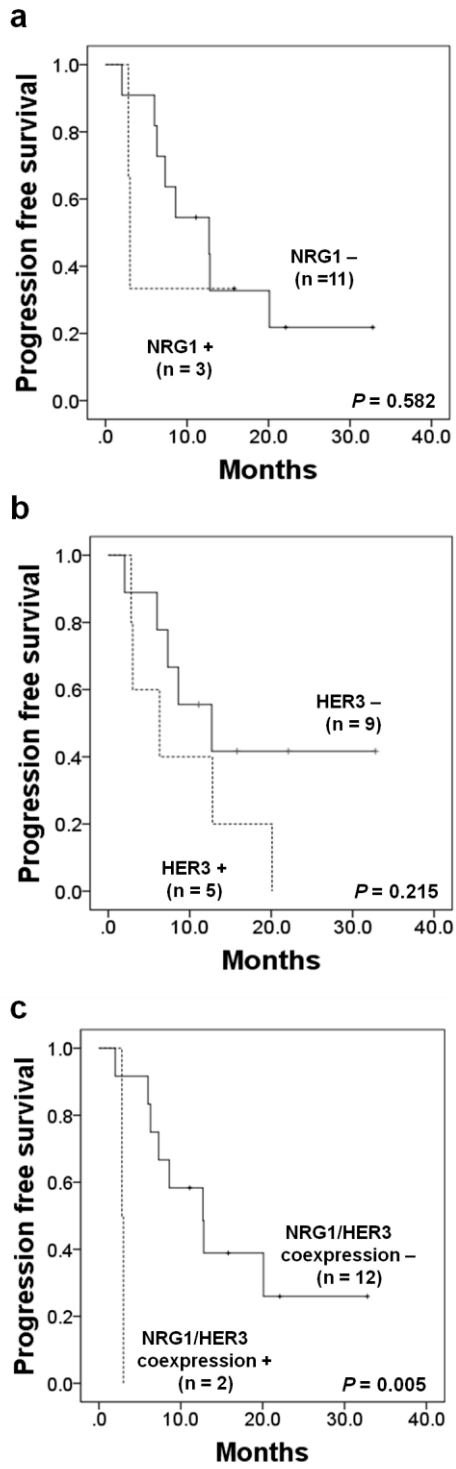
a
Progression free survival
1.0
0.8
0.6
0.4
0.2
0.0
.0
10.0
20.0
30.0
40.0
Months
NRG1 –
(n =11)
NRG1 +
(n = 3)
P = 0.582
b
Progression free survival
1.0
0.8
0.6
0.4
0.2
0.0
.0
10.0
20.0
30.0
40.0
Months
HER3 –
(n = 9)
HER3 +
(n = 5)
P = 0.215
c
Progression free survival
1.0
0.8
0.6
0.4
0.2
0.0
.0
10.0
20.0
30.0
40.0
Months
NRG1/HER3
coexpression –
(n = 12)
NRG1/HER3
coexpression +
(n = 2)
P = 0.005

Figure 9. Kaplan-Meier survival analysis accoridng to NRG1 and HER3 expression in HER2 positive and trastzumab treated GC patients. Progression free survival according to the expression of **a** NRG1, **b** HER3, and **c** NRG1/HER3 coexpression.

DISCUSSION

To date, the clinicopathologic role of NRG1 in GC has been unclear; therefore, we investigated the clinicopathologic implications and prognostic value of NRG1 expression in GC specimens. NRG1 overexpression was observed in 28.1% of GC samples, and NRG1 status was strongly associated with aggressive clinicopathologic parameters, including larger tumor size, infiltrative tumor border, lymphovascular invasion, neural invasion, lymph node metastasis, and advanced pathologic stage. Additionally, the overexpression of NRG1 predicted poor prognosis in patients with GC. To the best of our knowledge, this is the first study to demonstrate the clinicopathologic significance of NRG1 expression in a large-scale study of GC.

NRG1, a member of the NRG family, acts by binding to HER3 and HER4. HER3 is considered the major receptor for NRG1 [26, 27]. Recently, NRG1 has become the focus of research attention because of its overexpression in various cancers, including breast, urinary bladder, colorectal, prostate, and lung cancers [6]. In breast cancer, NRG1 overexpression was observed in approximately 30–80% of cases. In addition, NRG1 overexpression has been implicated in the activation of the HER3/HER2 signaling pathway, which mediates cancer cell proliferation, and other malignant features, including tumor invasion and metastasis [28–30]. Despite the increasing focus on NRG1 in various cancers, few studies have investigated the expression of NRG1 and

its association with clinical outcome in GC. Han et al. [23] reported that NRG1 overexpression was significantly related to advanced pathologic stage, lymph node metastasis, and poor prognosis; however, there have been several conflicting reports on the prognostic significance of NRG overexpression in various cancers [31–33]. Our results indicate that NRG1 overexpression is strongly associated with unfavorable clinicopathologic features in GC. Moreover, we identified pronounced differences between outcomes in GC patients with or without NRG1 overexpression. Hence, the results of the present study suggest that NRG1 overexpression may be an independent poor prognostic factor in GC.

Because of the close relationship between NRG1 and HER3, NRG1 expression has been suggested as a predictive biomarker for HER3 inhibition [6, 11]. In addition, NRG1 can promote resistance to HER2-targeted therapy through activation of HER3 and PI3K/Akt signaling both in vivo and in vitro [9, 34, 35]. Furthermore, a combination of anti-HER2 treatment with administration of a HER3 inhibitor has been proposed as a promising therapeutic strategy to improve tumor regression [36]. Therefore, our NRG1 expression and GCN results provide basic information of potential use for the development of clinical trials of HER3 inhibitor therapy and combined HER2 and HER3 inhibitor therapy. The expression and genetic status of NRG1 may facilitate identification of a GC patient subgroup who could benefit from anti-HER3 treatment.

Previous studies demonstrated that HER3 was overexpressed in the cytoplasm or membrane of tumor cells, which predicted poor prognosis in GC [15, 37, 38]. However, in our result, patients with cytoplasmic expression of HER3 have suffered slightly shorter DFS and DSS, without statistical significance ($P > 0.05$), and HER3 cytoplasmic expression did not correlate with lymph node metastasis or stage ($P > 0.05$). The survival analyses of HER3 expression may be affected by histologic subtypes and intracellular sublocalization (cytoplasmic vs. membranous). It may be additionally influenced by the sample size, race, ethnicity, antibody sources, and immunostaining protocol. Our results showed that HER3 cytoplasmic expression was significantly associated with HER2 positivity ($P = 0.022$) and the intestinal type of the Lauren classification ($P < 0.001$), and by subgroup analysis, HER3 cytoplasmic expression was associated with unfavorable prognosis in diffuse type GC ($P = 0.025$), but not in intestinal type ($P > 0.05$, data not shown), consistent with most previous studies [39, 40].

Interestingly, we found that GCs with membranous expression of HER3 showed significantly worse outcomes. In HER2 positive and trastzumab treated GC cohort, HER3 expression was found mainly on membrane, with or without cytoplasm of cancer cell, and all these five cases with HER3 expression suffered tumor progression. In Kaplan-Meier survival curve, HER3 expression group tended to show shorter PFS than in HER3 negative group. Based on these results, we suggest that HER3 membranous expression, not cytoplasmic localization, may have a significant role in HER2 positive and trastzumab treated GC patients.

Recent studies have also highlighted the clinical implications of HER4, since its expression is detected in various cancers [18, 41, 42]. Notably, HER4 has two conflicting roles in cancer. It can both inhibit and promote cell proliferation, depending on the localization of different HER4 isoforms generated by alternative splicing [18–20]. Alternative splicing of the HER4 gene leads to the production of two intracytoplasmic isoforms, CYT1 and CYT2. Compared with CYT2, translocation into the nucleus by CYT1 is less efficient. CYT1 also can induce the PI3K/Akt pathway, leading to increased cell proliferation and inhibition of cell differentiation [19, 43]. Depending on the presence of these isoforms, HER4 may show different intracellular localizations and varying clinical significance in malignancies. Previous studies on HER4 expression in GC failed to demonstrate a significant association with patients survival [15, 39], and little is known about the function of NRG1 in relation to the subcellular distribution of HER4 in GC. In a review of breast cancer studies, while HER4 cytoplasmic expression was favorably associated with patient survival, the significance of HER4 expression localized to the nucleus with regard to survival was uncertain [18]. In the current analysis, we evaluated HER4 nuclear and cytoplasmic expression independently in GC, according to the localization of immunostaining. We found that HER4 nuclear expression was tightly associated with favorable clinicopathologic features and better survival rates in GC; however, HER4 cytoplasmic expression failed to show a significant association with these parameters, in contrast to the reported results for this

protein in breast cancer. Moreover, NRG1 expression was tightly related to cytoplasmic expression of HER4 and exhibited an inverse association with HER4 nuclear expression, with borderline statistical significance. Considering the conflicting role of HER4 in cancer, our findings suggested that HER4 nuclear rather than cytoplasmic expression might be related to favorable clinical characteristics.

Our results demonstrate that *NRG1* amplification is a relatively rare event (0.5%) in GC. This is consistent with the findings of a previous study, which demonstrated that *NRG1* amplification is infrequent in GC [23]; however, alterations in *NRG1* GCN have not previously been investigated in GC. Despite the lack of acknowledged consensus criteria for GCN gain, our results revealed that this phenomenon was observed with relatively low frequency (14.7%). Additionally, we compared NRG1 protein expression and gene status. A significant discrepancy between *NRG1* GCN alteration and protein expression was identified, with cancer cells exhibiting *NRG1* amplification found to be negative for NRG1 immunostaining. One possible explanation for this discrepancy is that NRG1 may be overexpressed through mechanisms other than GCN alteration or gene amplification.

Our study has some limitations, including sampling bias of TMA slides, the use of a single institute retrospective cohort, and a lack of inclusion of patients receiving HER3 inhibitor therapy. Therefore, further comprehensive studies and clinical trials are necessary to clarify the usefulness of NRG1 for the

identification of cases where anti-HER3 treatment would be appropriate.

In conclusion, we evaluated the clinical significance of NRG1 and its receptors, including HER3 and HER4, in a large cohort of patients with GC. NRG1 was frequently overexpressed, and its expression was highly correlated with the cytoplasmic expression of HER3 and HER4 in GC. We also identified a strong correlation between high levels of NRG1 protein expression and increased *NRG1* GCN. Moreover, overexpression of this protein was significantly associated with aggressive behavior of GC including poor prognosis. However, prognostic significance of the expression of HER3, HER4 according to the intracellular sublocalization, was uncertain. These results suggest that NRG1 overexpression may predict poor clinical outcome and that targeting NRG1 represents a therapeutic opportunity in GC.

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국문 초록

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Neuregulin 1 (NRG1)은 HER3와 HER4의 대표적인 리간드로, 활성화를 통해 암을 유발하거나 전이를 촉진할 수 있다. 이번 연구는 위암에서 NRG1와 그 수용체인 HER3 및 HER4의 임상병리학적 의미를 규명하려고 하였다. 이를 위하여 502 개의 위암조직에서 면역조직화학검사를 통해 NRG1, HER3, HER4의 단백 발현을 조사하였다. 면역조직화학검사, RNA 제자리부합검사 (RNA *in situ* hybridization) 기법을 이용하여 HER2 상태, EBV 발현양상을 평가하였고, 현미부수체 불안정성 (MSI) 상태를 조사하였다. 또한 평가가 가능했던 388 개의 위암 조직에서 형광제자리부합검사 (Fluorescence *in situ* hybridization) 기법을 통해 *NRG1* gene copy number (GCN)를 조사하였다. 분석 결과에서 NRG1 단백 과발현은 28.1%에서 관찰되었고, HER3, HER4 단백 과발현과 유의한 연관성을 보였다. NRG1 단백 과발현은 위암에서 공격적인 특성, 즉 침윤성 경계, 림프관전이, 혈관전이, 신경전이, 진행된 병기 및 나쁜 예후와 강한 연관성을 보이고 있었지만, EBV, MSI, HER2 상

태와는 유의한 관계를 찾을 수 없었다. 다변량 생존 분석을 통해 NRG1 단백 과발현은 위암에서 독립적으로 나쁜 예후를 예측할 수 있음을 확인할 수 있었다. HER3 단백 과발현은 31.3%는 세포질에서 관찰되었으며, 2.6%에서는 세포막에서 관찰되었다. HER3 세포질 과발현이 예후와 연관성을 보이지 않았던데 반해, 세포막 과발현을 보이는 경우 그렇지 않은 경우에 비해 짧은 생존기간을 보였다. HER4 단백 과발현은 55.2%에서 세포질에, 22.9%에서 핵에서 관찰되었다. HER4의 핵 내 과발현은 세포질 과발현과 달리, 암의 낮은 악성도와 연관되어 있었고, 생존 분석에서도 핵 내 발현한 환자군이 그렇지 않은 군에 비해 좋은 예후를 보였다. *NRG1* GCN gain (GCN ≥ 2.5) 은 *NRG1* 유전자 증폭 (amplification)을 보이는 두 케이스를 포함하여 14.7%에서 관찰되었으며, NRG1 단백 발현과 중등도의 일치율을 보였다 (κ, 0.459; $P < 0.001$). HER2 양성소견을 보이며 trastzumab을 포함한 항암화학요법을 받은 14케이스의 위암조직에서 NRG1과 HER3 단백발현을 평가하였을 때, 두 케이스에서 NRG1과 HER3 동시발현을 보이고 있었으며, 이 두 케이스는 동시발현이 없는 다른 그룹에 비하여 유의하게 좋지 않은 예후와 연관성을 보였다. 결론적으로, 위암에서 HER3 및 HER4 단백 과발현과 달리, 그 리간드인 NRG1의 단백 과발현은 암의 악성도와 관련성을 보였고, 독립적으로 나쁜 예후를 예측하는 인자로 사용할 수 있는 가능성을 확인하였다.

주요어: 위암, Neuregulin1, 면역조직화학검사, 형광제자리부합검사, Copy number gain

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